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Divergent functions of *orthodenticle*, *empty spiracles* and *buttonhead* in early head patterning of the beetle *Tribolium castaneum* (Coleoptera)

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ABSTRACT

The head gap genes *orthodenticle* (*otd*), *empty spiracles* (*ems*) and *buttonhead* (*btd*) are required for metamerization and segment specification in *Drosophila*. We asked whether the function of their orthologs is conserved in the red flour beetle *Tribolium castaneum* which in contrast to *Drosophila* develops its larval head in a way typical for insects. We find that depending on dsRNA injection time, two functions of *Tc-orthodenticle1* (*Tc-otd1*) can be identified. The early regionalization function affects all segments formed during the blastoderm stage while the later head patterning function is similar to *Drosophila*. In contrast, both expression and function of *Tc-empty spiracles* (*Tc-ems*) are restricted to the posterior part of the ocular and the anterior part of the antennal segment and *Tc-buttonhead* (*Tc-btd*) is not required for head cuticle formation at all. We conclude that the gap gene like roles of *ems* and *btd* are not conserved while at least the head patterning function of *otd* appears to be similar in fly and beetle. Hence, the ancestral mode of insect head segmentation remains to be discovered. With this work, we establish *Tribolium* as a model system for arthropod head development that does not suffer from the *Drosophila* specific problems like head involution and strongly reduced head structures.

Introduction

The arthropod head is built by several segment primordia that fuse to form the rigid head capsule with limbs that are adapted to different roles in feeding (Snodgrass, 1935; Weber, 1966). Curiously, the number of head segments and the potential contribution of non-segmental tissue (Rempel, 1975; Scholtz and Edgecombe, 2006) as well as the evolution of the labrum remain disputed (Budd, 2002; Haas et al., 2001). Also, the developmental genetics of insect head patterning remain enigmatic because head involution of *Drosophila melanogaster* leads to a derived and reduced larval head morphology that has hampered mutant analysis (Fig. 1) (Nassif et al., 1998; Dalton et al., 1989; Jürgens et al., 1986).

The gnathal segments (mandible, maxilla and labium) constitute the posterior portion of the arthropod head. Except for the mandible (Vincent et al., 1997), they are patterned like the other trunk segments in *Drosophila* according to the well established hierarchical segmentation cascade involving maternal coordinate genes, gap-, pair rule- and segment polarity genes (St Johnston and Nusslein-Volhard, 1992). Segment identity specification is accomplished by the Hox genes (McGinnis and Krumlauf, 1992). Anterior to the mandible, however, no pair rule patterning is observed and the anterior most expression of a gene of the Hox cluster is in the intercalary segment (Bucher and Wimmer, 2005; Diederich et al., 1991). For patterning of anterior head segments, *Drosophila* makes use of the so-called head gap like genes *otd*, *ems* and *btd* (Cohen and Jürgens, 1991; Cohen and Jürgens, 1990). They are expressed early in embryogenesis in broad and overlapping domains (Dalton et al., 1989; Finkelstein and Perrimon, 1990; Walldorf and Gehring, 1992; Wimmer et al., 1993, 1997). Also their deletion domains are overlapping and correspond roughly to the expression patterns. As judged by cuticular phenotypes as well as *engrailed* and *wingless* (*wg*) expression in mutants, loss of *otd* function leads to a loss of the ocular and the antennal segments. *ems* is required for the posterior most parts of the ocular segment as marked by the absence of the *engrailed* head spot but presence of the adjacent ocular *wg* domain (head blob). In addition, *ems* mutants lack antennal and intercalary segments (Cohen and Jürgens, 1990) and the anterior portion of the mandibular segment (Dalton et al., 1989; Walldorf and Gehring, 1992). Mutations in *btd* lead to loss of antennal, intercalary, mandibular and the anterior portion of the maxillary segments (Wimmer et al., 1996). The head gap genes are not required for patterning the labrum (Cohen and Jürgens, 1990, 1991; Wimmer et al., 1996). Their regulation depends on three maternal systems. Via *bicoid* the anterior system activates *otd*, *ems* and *btd*, the terminal system is required for anterior positioning of both *otd* and *btd* and the dorso-ventral system provides further pattern refinement (Dalton et al.,

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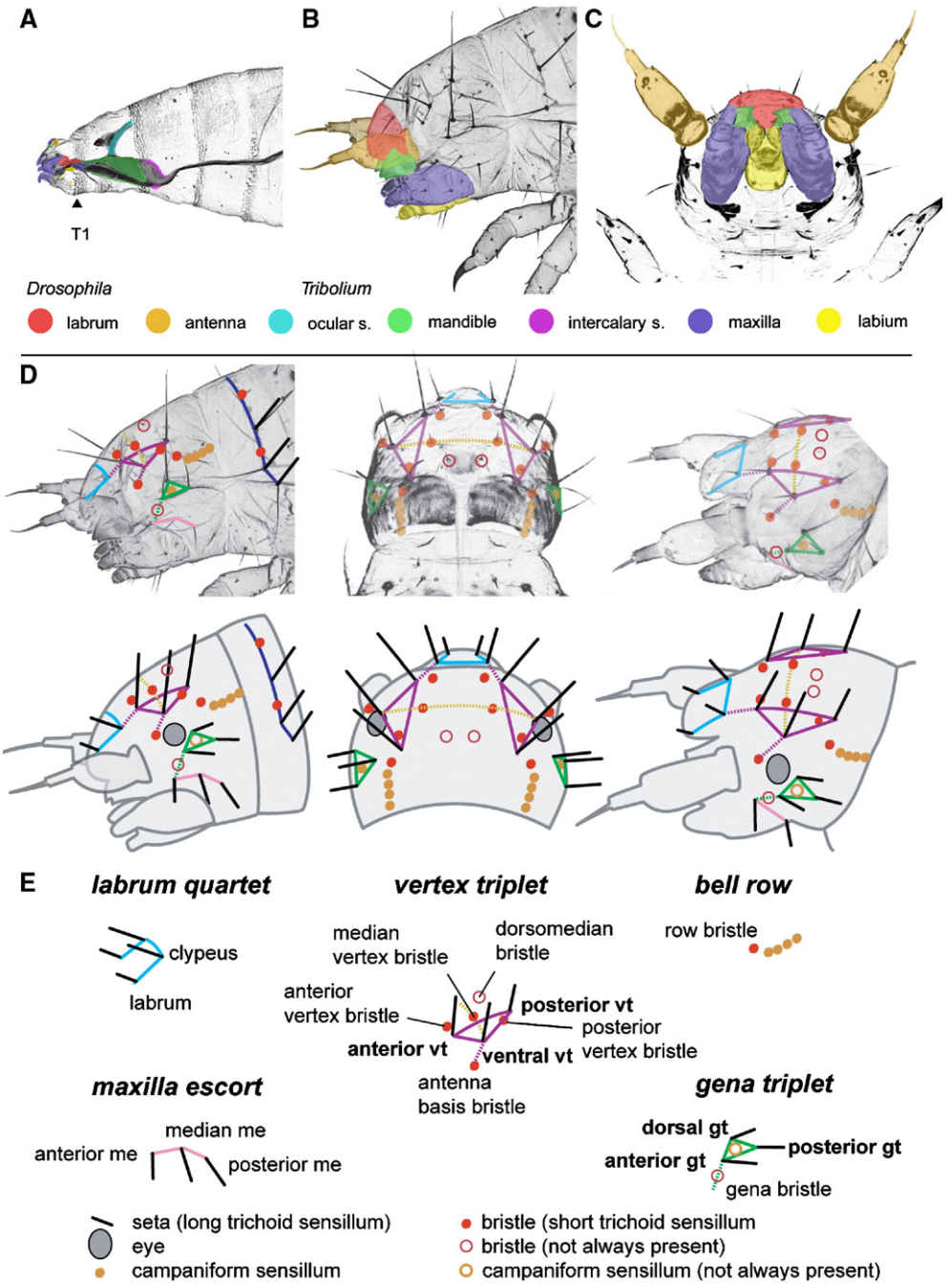

Fig. 1. Head cuticles of *Drosophila* and *Tribolium*. (A) The *Drosophila* larval head is involuted into the thorax and its structures are heavily reduced. (B, C) The *Tribolium* larva in contrast displays a head with all structures typical for insect heads (B: lateral view; C: ventral view; color code for head appendages is the same in A–C). (D) The dorsal and lateral portions of the *Tribolium* head are marked by a pattern of setae and bristles. Groups of setae are connected by colored lines. The angles of the colored lines mark the position of the long setae while red dots indicate short bristles. Orange dots indicate campaniform sensillae while open circles mark bristles/campaniform sensillae that are not found on all wild-type cuticles. (E) For future reference, setae and bristles have been grouped and been given names. Note that neither color code nor names indicate any developmental or segmental units. See text for details.

1989; Finkelstein and Perrimon, 1990; Gao and Finkelstein, 1998; Gao et al., 1996; Grossniklaus et al., 1994; Wimmer et al., 1995). This activation by maternal factors together with the gap phenotypes has led to their classification as gap like genes. The suggestion that their combinatorial action would also specify segmental identity (Cohen and Jürgens, 1991; Grossniklaus et al., 1994) has not proven correct (Gallitano-Mendel and Finkelstein, 1998; Wimmer et al., 1997). *ems* but not *otd* has some homeotic selector function and requires *btd* to do so (Gallitano-Mendel and Finkelstein, 1998; Schöck et al., 2000).

Strikingly, in vertebrates the orthologs of *otd* and *ems* are expressed in the anterior brain Anlage and are required for its development (Acampora et al., 1998; Reichert and Simeone, 1999; Simeone et al., 1992; Treichel et al., 2003; Wimmer et al., 1993). Cross-phylum experiments have shown that the murine Otx and Emx2 proteins are able to partially rescue respective *Drosophila* mutant brain phenotypes (Hartmann et al., 2000; Leuzinger et al., 1998). The *Drosophila* Otd protein in turn has an activity similar to the endogenous ortholog in *Xenopus* (Lunardi and Vignali, 2006) and mouse brain development (Acampora et al., 1998). Together with other data, this has led to the view of an urbilaterian origin of the animal brain and of highly conserved brain pattering mechanisms (Denes et al., 2007; Lichtneckert and Reichert, 2005; Reichert and

Simeone, 2001; Tessmar-Raible et al., 2007). However, the murine SP factor that had previously been described as buttonhead (*mBtd*) (Treichel et al., 2003) actually belongs to the SP8 family (Beermann et al., 2004; Griesel et al., 2006). Hence, the striking functional similarities of murine *mBtd* and *Drosophila btd* became arguable while the true vertebrate ortholog of *Dm-btd* has remained unclear.

With respect to head development, *Drosophila* is a poor representative of the arthropods because of its highly derived mode of larval head development. In contrast to *Drosophila*, the red flour beetle *Tribolium* develops a regular larval head with all structures typical for an insect head (Bucher and Wimmer, 2005). With robust RNAi techniques established (Brown et al., 1999; Bucher et al., 2002; Tomoyasu and Denell, 2004) and with the genome sequenced (The Tribolium Genome Sequencing Consortium, in press) *Tribolium* has evolved into an arthropod model system second only to *Drosophila*.

With this work, we introduce *Tribolium* as a model for larval head development in arthropods. First we describe the bristle pattern of the larval head in order to provide landmarks for mapping patterning defects. Then we analyze the orthologs of the *Drosophila* head gap like genes *Tc-otd1*, *Tc-ems* and *Tc-btd*. We find that the latter two do not function as head gap genes and that changes in gene function correlate with altered expression patterns. Our data suggest that cross-phyla comparisons of gene function should not be based on highly variable early patterning processes.

Materials and methods

Phylogenetic analysis

The zinc-finger and buttonhead boxes of Tc-SP8, *Drosophila* and mouse SP-factors were aligned using ClustalW with subsequent manual curation. Only clearly aligned positions were used for the phylogenetic analysis. A phylogenetic tree was calculated using the Tree-Puzzle algorithm (Schmidt et al., 2002) at http://bioweb.pasteur.fr/seqanal/interfaces/Puzzle.html with standard options but "calculation of clock-like branch lengths" and the "more exact algorithm". The resulting tree was visualized using TreeView 1.6.6. (R.D.M. Page 2001). To identify additional conserved motifs, the mouse Sp5, Dm-Btd and Tc-Btd proteins were subjected to pair wise dot blot analysis at SRS using standard settings. The subsequent ClustalW alignment as implemented in the MEGA 3.1 (Kumar, Tamura, Nei) did not properly align all domains identified in the dot blots and were hence curated manually as well as the N-terminal part of the alignment. The alignment was displayed by "boxshade" at www.ch.embnet.org/software/BOX_form.html.

Mouse Emx1/2 and Dm-Ems were retrieved from the SRS protein database. tBlastn analysis using Tc-Ems as query in the *Drosophila* genome revealed the *ems*, *e5* and the *ex ex* genes as best hits. Blast of Dm-E5 in *Tribolium* retrieved Tc-Ems, Tc-Bagpipe and Tc-Exex. Pair wise dot blot analysis and ClustalW alignments, calculation and representation of the tree and alignment depiction were done as described above. As Mm-Emx1 and Mm-Emx2 appeared very similar in the dot blot analysis, only Mm-Emx1 was used for the analysis.

Double stainings

False color representation of double fluorescence images was performed as described in (Wohlfrom et al., 2006). Note that the NBT/BCIP staining quenches the fluorescent fastRed staining to some extent such that weak overlaps may be missed.

RNAi

Templates were prepared by PCR with T7-primers from plasmid template comprising full length (1.1 kb) plus 125 bp 5′UTR and 700 bp 3′UTR of the *Tc-otd1* and 520 bp of the open reading frame plus 200 bp 3′UTR of *Tc-ems*. DsRNA was produced using the Megascript Kit (Ambion). Concentrations for parental RNAi were 2–4 μg/μl (*Tc-otd1*), 2.5 μg/μl (*Tc-ems*) and 1–5 μg/μl (*Tc-btd*) and for embryonic RNAi 0.1–1 μg/μl (*Tc-otd1* and *Tc-btd*) and 0.5 μg/μl (*Tc-ems*). Injections were performed as described (Brown et al., 1999; Bucher et al., 2002). To test for the portion of embryos that do not develop cuticle, we injected 3.7 μg/μl *Tc-otd1*-dsRNA and collected eggs of injected and non-injected animals three times, respectively. Of both hatching and non-hatching larvae, cuticles were prepared. The number of eggs in the egg collection was counted as well as the portion of developed cuticles and empty eggshells without visible remnants of cuticles (wt: $n=238$, Tc-otd1RNAi: $n(d7)=31$ $n(d8)=45$ $n(d10)=106$). The experiment was done three times using two independently cloned *Tc-otd1* templates.

Microscopy

Cuticle preparations were documented by laser scanning microscopy as described before (Wohlfrom et al., 2006). For the presentation, the colors have been inverted using Photoshop 7.0 (Adobe). Nomarski optics and fluorescent images of the whole mount in situ stainings were documented using a Zeiss Axioplan microscope (Zeiss, Jena).

Testing negative results for Tc-btd

Pupae were injected with 5 μg/μl dsRNA comprising the entire coding region (756 bp). A small portion of the egg collection (7 days after injection) was used for cuticle preparations, the rest was fixed. Whole mount in situ staining against *Tc-btd* including *Tc-caudal* as positive control in the same color reaction revealed no detectable *Tc-btd* activity but normal *Tc-caudal* expression in most embryos. All cuticles of this egg collection were wild type and the portion of empty egg shells was normal. To test for potential later Tc-Btd function, we also allowed eggs of injected pupae to develop. The ratio of hatching larvae was within the normal range (6 of 19 injected as compared to 10 of 21 in the buffer control) and all hatched larvae developed to morphologically normal adult beetles. Also injection of dsRNA into embryos did not cause overt phenotypes.

Results

The Tribolium L1 larval head

For better interpretation of cuticular phenotypes, we have determined a set of cuticular structures that mark different regions of the *Tribolium* head. The insect head is a complex structure that arose by the fusion of the proposed acron–an anterior non-segmental tissue (i.e. not serial homologous to trunk segments)–and several segments that are serial homologous to trunk segments. Some authors suggest that the acron is minuscule or even absent (Schmidt-Ott and Technau, 1992). The *Tribolium* larva is prognath, i.e. the mouth opening is oriented toward anterior. The mouth appendages encircle the mouth opening forming a preoral cavity. This "ring" is closed posteriorly by the labium and laterally by mandibles and maxillae. These gnathal appendages are markers for the posterior portion of the respective segments as they arise from *Tc-engrailed/Tc-wingless (Tc-wg)* positive tissues. The antennae are oriented toward anterior and are a marker for the posterior portion of the antennal segment. The larval eyes are located posterior to the antennae with respect to the larval anterior–posterior-axis (ap-axis). They reside below the cuticle and their position is not marked by any specific cuticular structure. Before clearing of cuticles, however, they can be identified within the head capsule and used as markers for part of the ocular region. The clypeolabrum is located between the two antennae and projects downward to encircle the preoral cavity from the front. The basis of the clypeus probably reaches the dorsal head (vertex). The articulation that separates clypeus from labrum is not always visible in L1 larvae. A set of sensory organs provide cuticular markers for the lateral and dorsal sides of the *Tribolium* L1 larval head (Figs. 1D–E). In 62 wild-type cuticles, the entire set could be identified proving a high degree of constancy and reproducibility (exceptions are indicated below and are marked by open circles in Fig. 1). In the following, all bristles of one side of the head are described in an order that facilitates orientation—their names are quoted when mentioned for the first time.

At the posterior rim of the head at a dorso-lateral position, a row of four (in most cases) campaniform sensillae projects anteriorly ("bell row"). Anteriorly, the bell row is delimitated by the "row bristle". Dorsal to the latter, the posterior most of a triangle of three setae is found ("vertex triplet") composed of a "posterior vt", "anterior vt" and "ventral vt". Close to the posterior vt, the "posterior vertex bristle" is located. The "median vertex bristle" is found on an imaginary line between the two ventral vt of both sides. Dorsal to the anterior vt, the "anterior vertex bristle" is found. On an imaginary line from the ventral vt toward the base of the antenna, the "antenna basis bristle" is located. The bristles of the "labrum quartet" are located on an anterior extension of a line that runs through the "posterior vt" and the "anterior vt". The "gena triplet" is found ventral to the vertex triplet and ventro-anterior to the bell row. It marks the lateral portions of the head (gena). It is composed of "anterior gt", "posterior gt" and "dorsal gt" and in many cases encloses a campaniform sensillum (open circle). In most cases, the "gena bristle" is found between the anterior gt and

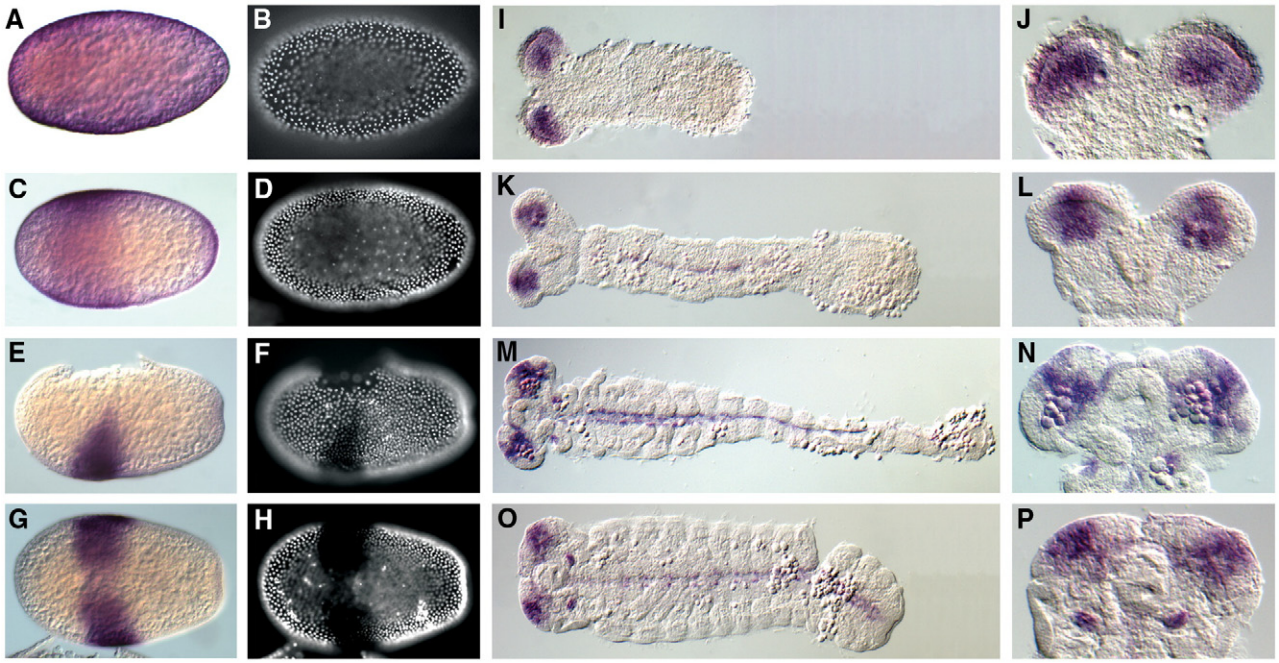

Fig. 2. Expression of *Tc-otd1*. (A, B) *Tc-otd1* is provided maternally. (C–H) The initially broad domain rapidly retracts from both poles and comes to lay at the anterior portion of the head. Panels E and F are lateral views, panels G and H are ventral views. (I, K, M, O) During germ band growth, the head domain remains rather stable while a midline domain arises de novo. (J, L, N, P) Close ups of the respective heads.

the maxilla. Three short setae line the maxillary bulge ("anterior, median and posterior maxilla escort") but depending on the orientation of the head they are sometimes difficult to identify on one side. The dorsal median region is poor in markers. The "dorsomedian bristles" are not visible on all wild-type cuticles. The larval eyes are located below the cuticle and we have not been able to detect a cuticular marker of the eye itself. However, in a lateral view, the eye appears in a field defined by four sensory organs: the ventral vt, the antenna basis bristle and the dorsal and anterior gt. In dorsal views, the eye appears below to the ventral vt.

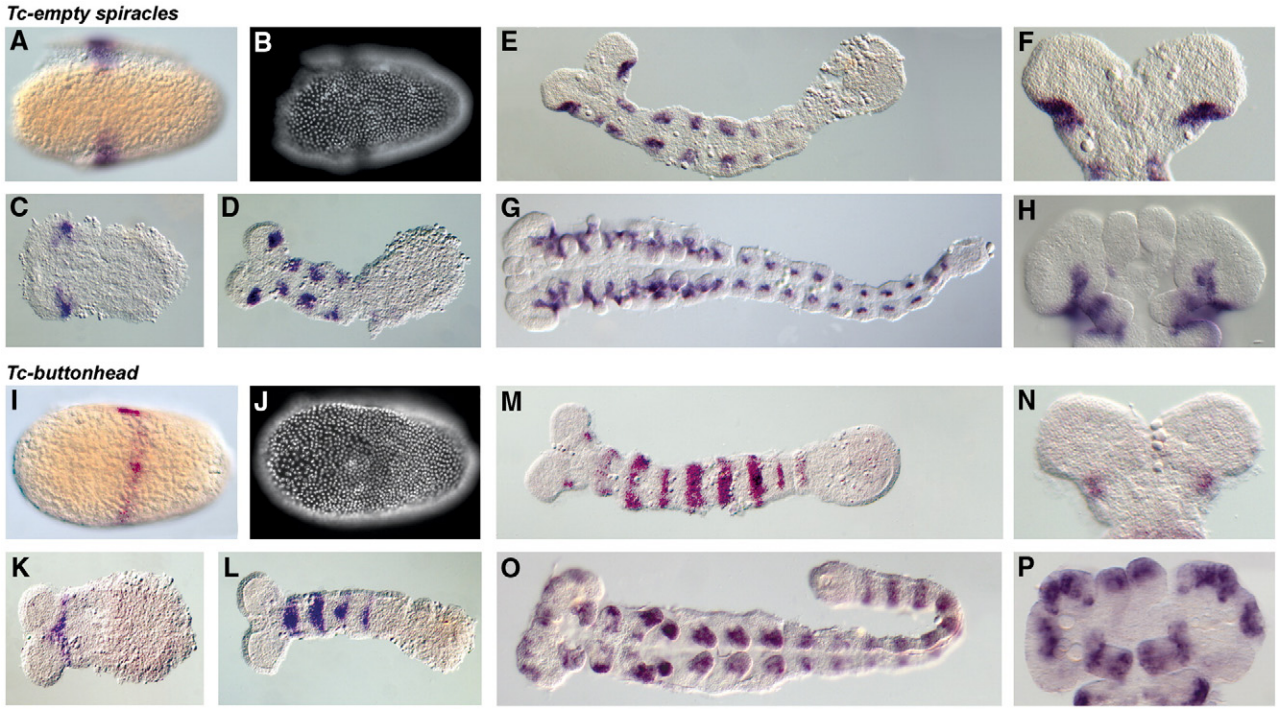

Fig. 3. Expression patterns of *Tc-ems* and *Tc-btd*. (A, B) *Tc-ems* starts to become expressed in the late blastoderm stage in a narrow stripe. (C) This stripe comes to lie in the anterior portion of the antennal segment anlagen. (D, E, G) During elongation, a segmentally reiterated pattern of lateral *Tc-ems* patches arises. (F, H) Close ups of the heads of embryos shown in panels E and G, respectively. (I, J) Also *Tc-btd* expression starts as a narrow stripe somewhat earlier than *Tc-ems*. (K) This stripe marks the future mandible. (L, M, O) During elongation, segmental stripes arise in anterior posterior sequence. (M, O) Midway through elongation, also the more anterior antennal and intercalary stripes appear and the appendages get *Tc-btd* positive. (N, P) Close ups of heads of the embryos shown in M and O, respectively. At late stages, *Tc-btd* becomes expressed in the labrum and several domains in the anterior head.

Identifying orthologs of orthodenticle, empty spiracles and buttonhead

Two *Tribolium* orthologs of the single *Drosophila otd* gene have been described (*Tc-otd1* and *Tc-otd2*) (Li et al., 1996). Expression of *Tc-otd2* starts only at the extended germ band stage when the head has already formed and is therefore unlikely to contribute significantly to early head patterning.

Using degenerated primers and scrutinizing the genomic sequence, we identified three SP factor genes. One being identical to the described *Tc-SP8* (Beermann et al., 2004). Phylogenetic analysis including all *Drosophila* and mouse SP factors reveals single *Tribolium* SP8 (Beermann et al., 2004) and *Drosophila* SP8 (originally termed *D-Sp1*; Wimmer et al., 1996; Schöck et al. 1999) orthologs to the mouse SP8/9 family that probably also includes the SP7 gene (Fig. S1D). *Dm-* and *Tc-SP1234* are likely orthologs to the mouse *Sp1/2/3/4* genes. The mouse *SP5* gene is the closest related gene to the third *Tribolium* SP-factor (called Tc-Btd) but orthology is not unequivocal based on sequence analysis restricted to the zinc finger and *buttonhead* box. Supporting this assumption, however, 11 amino acids of the SP-box are identical between Tc-Btd and SP5 but 5 maximum with the other mouse SP-factors, respectively (not

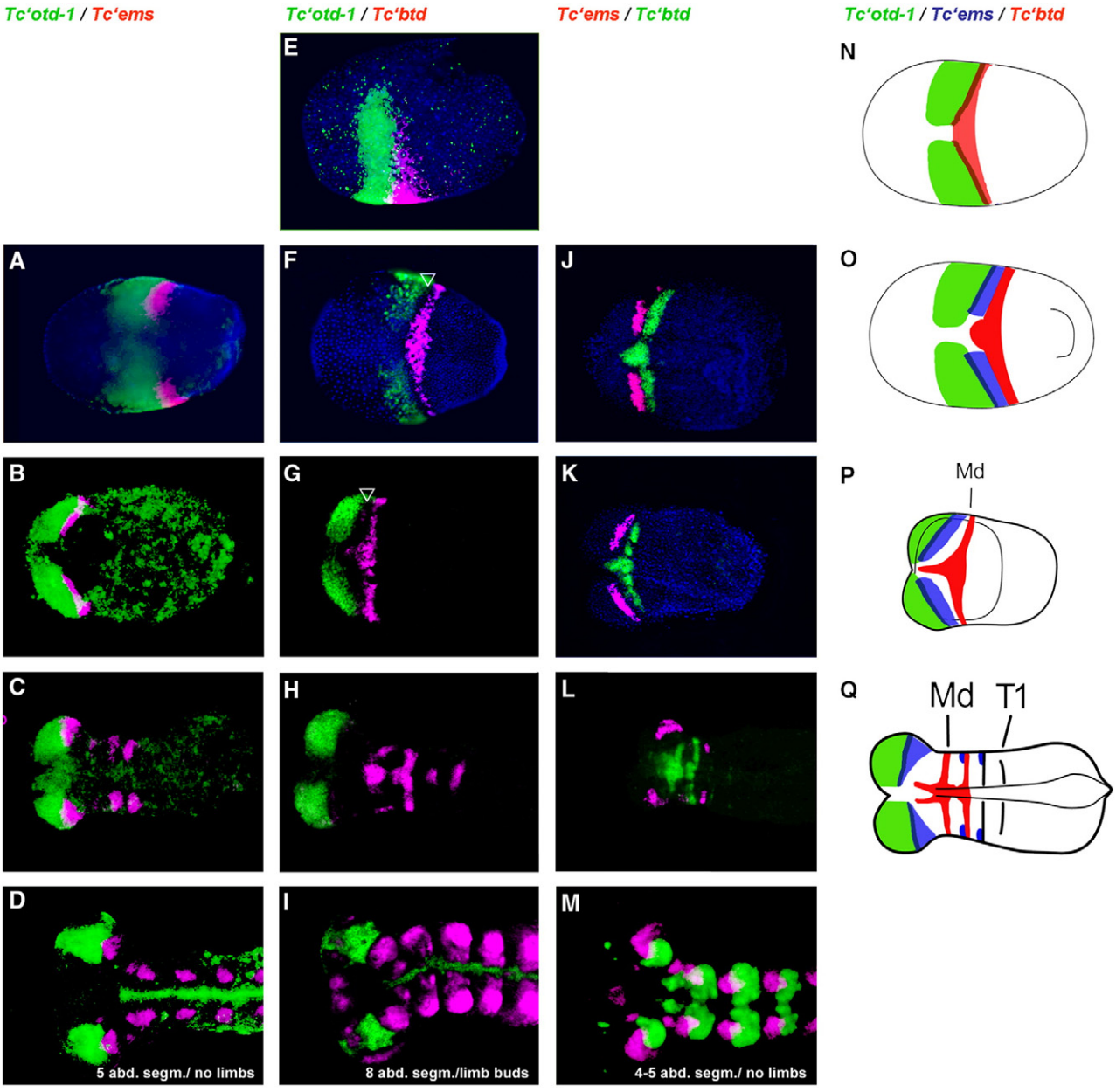

Fig. 4. Double in situ hybridization of *Tc-otd1*, *Tc-ems* and *Tc-btd*. False color representation of double stainings combining a conventional with a fluorescent staining. In all panels, anterior is to the left, all but panel E are ventral views. Comparable stages are shown in one row. Exceptions are panel J which is slightly older than panels A and F and D which is much younger than panels I and M. (A–D) *Tc-otd1* in green and *Tc-ems* in red. *Tc-ems* expression starts at a late blastoderm stage adjacent to *Tc-otd1* with a small overlap (A). This relative position including overlap remains constant throughout initial elongation (B, C). Later, *Tc-ems* expression fades at the lateral sides such that the overlap with *Tc-otd1* remains only in medial parts of its domain (D). (E–I) *Tc-otd1* in green and *Tc-btd* in red. (E) *Tc-btd* expression starts earlier than *Tc-ems* and only in its very first stages shows some overlap with *Tc-otd1*. (F) Slightly later, the stripe has become even narrower and the overlap with *Tc-otd1* is lost. In the opening clearance, *Tc-ems* will arise (compare open arrowhead in F with A and J). (G, H) The *Tc-btd* stripe comes to lay in the mandibular segment and the clearance to the *Tc-otd1* domain becomes broader (see open arrowhead). (I) At later elongation stages, *Tc-btd* stripes are found also in intercalary and antennal segments closing the gap to *Tc-otd1* again. (J–M) *Tc-ems* expression shown in red and *Tc-btd* in green. (J) In late blastoderm and early germ rudiment stages (shown in panel J), *Tc-btd* and *Tc-ems* expression is adjacent, probably without overlap. (K, L) In subsequent stages, the gap between these expression domains broadens. (M) Later, segmental expression is similar in all segments and overlap is observable in each segment. (N–Q) Schematic representation of our analysis with *Tc-otd1* shown in green, *Tc-btd* in red and *Tc-ems* in blue.

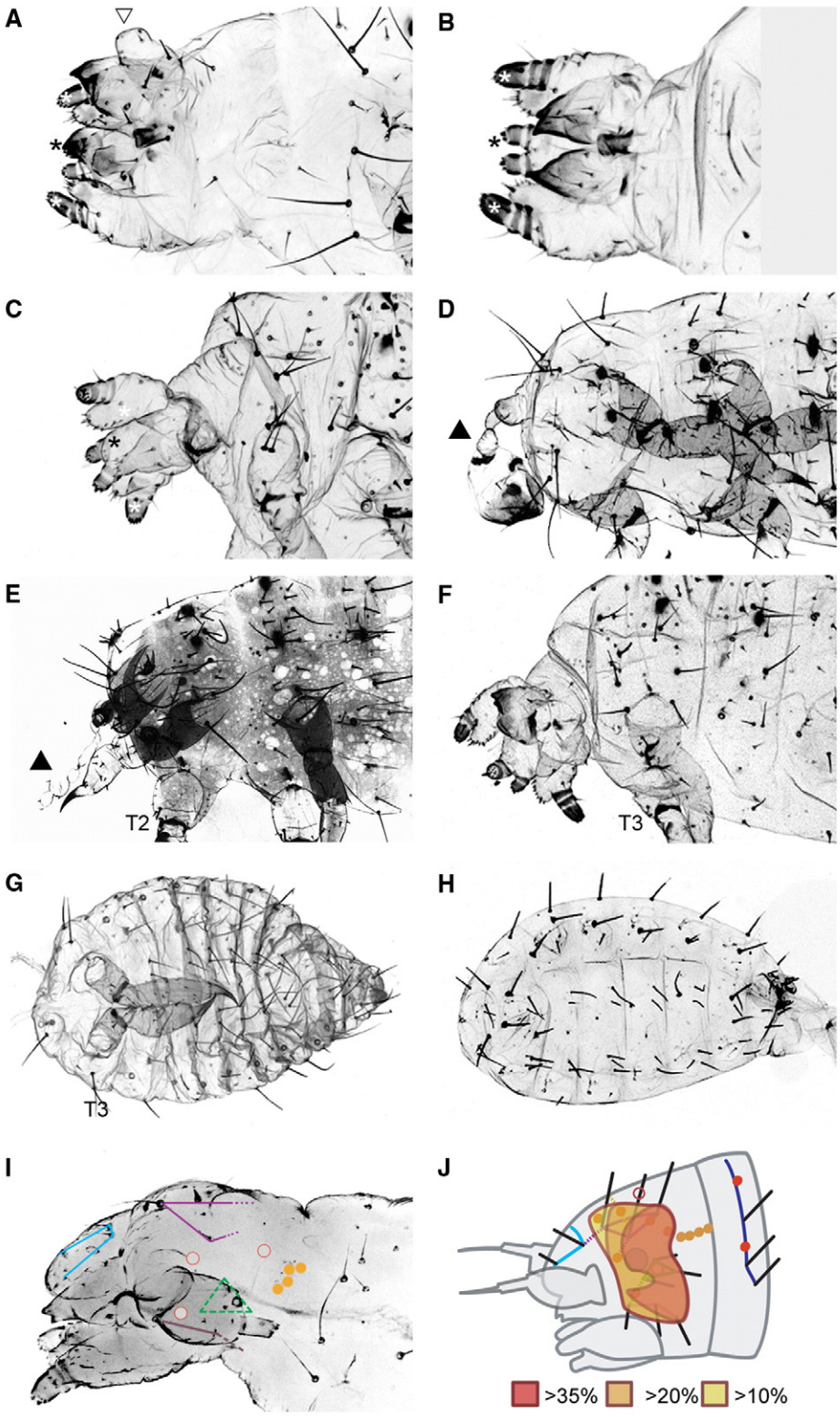

Fig. 5. *Tc-otd1* RNAi cuticle phenotype. The *Tc-otd1* phenotypic series ranges from deletions of single bristles to loss of entire head and thorax. The labium and the maxillae are marked by black and white stars, respectively. (A) Dorsolateral view of an intermediate phenotype that has lost the antennae and the entire dorsal and lateral setae but retains the labrum (open arrowhead). (B) Dorsal view of a phenotype where all head structures but the gnathal appendages are deleted. (C) Lateral view of a cuticle which only retains labium and maxillae. (D) Ventrolateral view on a cuticle the head of which is reduced to a cuticular tube (black arrowhead). (E, F) In stronger phenotypes, also the thoracic segments are affected. More anterior structures are reduced to a cuticular tube (E). In rare cases, some gnathal segments remain but thoracic segments are lost (F). (G, H) Strong phenotypes lack the entire head and parts (G) or the entire thorax (H). Some knockdown embryos loose any sign of segmentation and form cuticular sacs decorated with some bristles (not shown). (I, J) Weak phenotypes that retain antennae and labrum were scored for the head bristle pattern. (I) Labrum and distorted antennae are present. Missing setae are posterior vt, all three gena triplet setae and the posterior maxilla escort. Missing bristles are marked by red circles. (J) Schematic summary of the analysis. All bristles and setae that were lost in >35%, >20% and >10% are combined in colored fields, respectively (n=28). Because additional markers are lacking, the extension of these fields has been chosen to just comprise the missing bristles. A cuticle field extending from the lateral head to the dorsum in the middle of the head is most sensitive. Less affected are regions anterior to this field. Detailed results for the bristle analysis are found in Table S1.

shown). Even more equivocal is the association of *Drosophila* Btd with other SP-factors, maybe due to the high evolutionary rate of Dm-Btd (see long branch in Fig. S1D). We see only remnants of the SP-box in Dm-Btd (Fig. S1A). However, the genomic location of both *Drosophila* and *Tribolium btd* genes close to their SP8 paralogs (Schöck et al., 1999; The Tribolium Genome Sequencing Consortium, in press), respectively, and their similar expression patterns (see below) argue in favor of orthology. Therefore, we are convinced that *Tc-btd* is the true ortholog of *Dm-btd* (see Fig. S1A for additional findings).

Tc-ems was isolated in a library screen using the *Drosophila* sequence as a probe (Hausdorf, 1996). No additional paralog is found in the genomic sequence. In the *Drosophila* genome, however, an additional gene with a high similarity is present, *Dm-E5* (CG 9930). Our phylogenetic analysis reveals Tc-Ems as the single ortholog to the paralogs Dm-E5 and Dm-Ems (Fig. S1C). The *Drosophila* gene pair probably arose by duplication after the separation of Dipteran and Coleopteran ancestors. The mouse paralogs *emx1* and *2* have probably arisen by a recent independent duplication event (Williams and Holland, 2000). Interestingly, the length of Tc-Ems is more similar to the short mouse proteins rather than to the much longer *Drosophila* proteins. These *Drosophila* specific expansions have most likely occurred independently because they are located at different locations between box 1 and 2 (Dm-Ems) and at the C-terminus (Dm-E5). *Dm-E5* is not transcribed in blastoderm stages but becomes expressed in a segmentally reiterated pattern similar to late *Dm-ems* expression from stage 10 onward (Williams and Holland, 2000) and is therefore not relevant for our discussion.

Different onset of expression of the head gap gene orthologs

Tc-otd1 expression has been described before (Li et al., 1996; Schröder, 2003). It differs from *Dm-otd* by its ubiquitous maternal contribution (Figs. 2A, B). During advancing blastoderm stages, this expression retracts from both poles clearing the anlagen of the extraembryonic tissues (anterior) and the posterior portion of the embryo (Figs. 2C–H) and also from ventral tissue (Fig. 2G). From this stage on, the head expressions of *Dm-otd* and *Tc-otd1* are very similar. At later stages, activity along the midline and in the anterior portion of the mandibles arises (Figs. 2K–P).

Expression of *Tc-ems* starts at the late blastoderm stage, when extraembryonic tissue and germ rudiment become morphologically distinguishable (Figs. 3A, B). This stripe is narrow and sharp from the beginning and remains adjacent to the ocular *Tc-wg* stripe in germ bands without detectable overlap. Also the later arising antennal *Tc-wg* stripe touches *Tc-ems* expression without overlap (not shown). This locates the first *Tc-ems* domain to the anterior portion of the antennal and the posterior most portion of the ocular segment. The non-overlapping adjacent expression of *wg* and *ems* is also seen in the head ectoderm of *Drosophila* stage 10 embryos (Urbach and Technau, 2003a, b). During germ band growth, additional segmental patterns arise in the lateral portions of gnathal and trunk segments that appear similar to the *Drosophila* expression but are not further analyzed here (Figs. 3D, E, G).

Tc-btd expression starts in the late blastoderm stage as well but arises somewhat earlier than *Tc-ems* (Figs. 3I, J). In the differentiated blastoderm stage, it forms a narrow stripe that later comes to lie in the mandibular segment (Fig. 3K). Subsequently, segmental stripes arise (Figs. 3L, M, O). Rather late, expression appears also in the antennal segment (Fig. 3M) and the ocular region, the labrum and in the anterior head (Figs. 3O, P). Initially, the future mandibular *Tc-btd* stripe is separated from the ocular *Tc-wg* domain only by a few cells (not shown) but this gap extends significantly in subsequent stages.

By a series of double stainings, we asked whether *Drosophila*-like extensive overlap of expression patterns (Wimmer et al., 1997) is found in *Tribolium* (Fig. 4). Shortly before the extraembryonic tissue

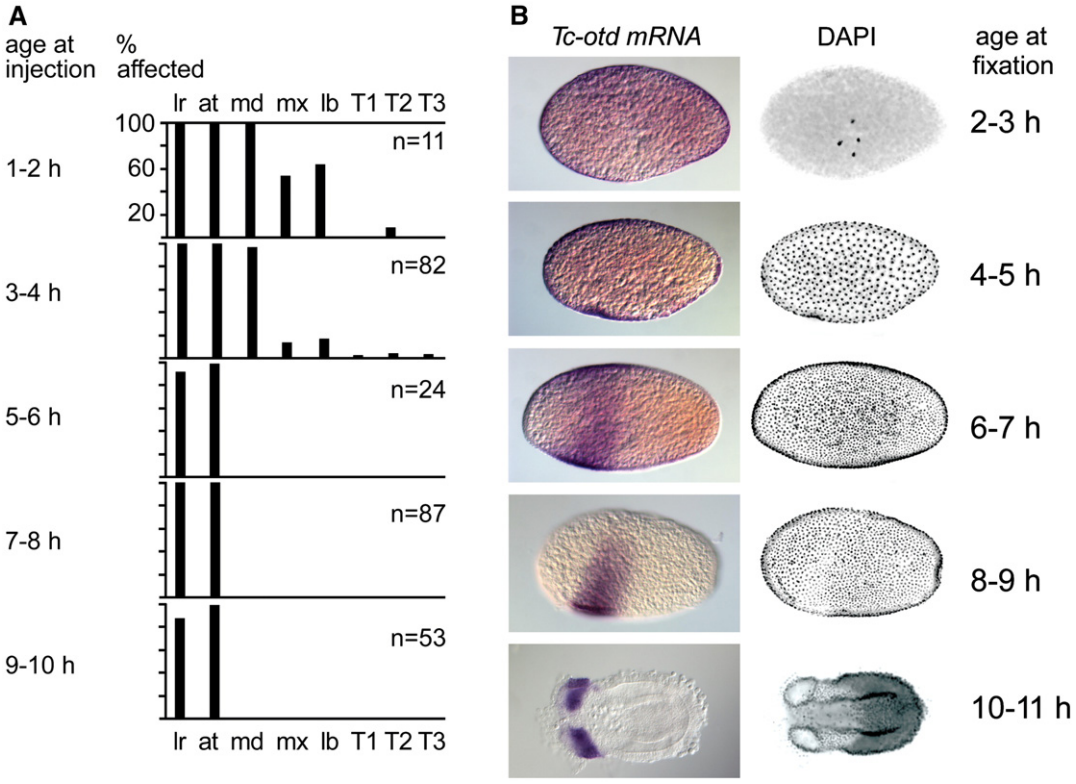

Fig. 6. Early regionalization defects depend on knock down of early *Tc-otd1* function. (A) Embryos were injected at different time points after egg collection and the cuticle phenotype scored for the presence of the head and trunk appendages. Only injections within the first 4 h of development (at 32 °C) lead to defects of gnathal and thoracic segments. Later injections lead only to loss of antenna, eye and labrum. (B) Staining of similarly staged embryos for *Tc-otd1* expression. Assuming a short delay between injection, mRNA degradation and protein decay, we find that the retraction of *Tc-otd1* expression to the anterior head (6–7 h) correlates with the more restricted deletion of antenna, eye and labrum.

begins to become morphologically visible, the mandibular *Tc-btd* stripe arises. Initially, it overlaps with the still broad *Tc-otd1* stripe (Fig. 4E) but shortly later, a gap arises between *Tc-otd1* and *Tc-btd* where the *Tc-ems* expression becomes detectable (Figs. 4F, A, J; J is slightly older than A, F). Initially, the newly formed *Tc-ems* and the *Tc-btd* stripes are adjacent but non-overlapping. Subsequently, the *Tc-btd* expression domain becomes separated from *Tc-ems* expression (Fig. 4, compare J with K). This gap broadens with time (Fig. 4L) leaving space for posterior antennal and intercalary segment anlagen. At mid elongation, the antennal and intercalary *Tc-btd* domains arise (Figs. 4I, M; I is older than M and D) that fill parts of the gap and lead to a repetitive pattern that is similar from antenna to posterior trunk segments. From that stage on, *Tc-btd* and *Tc-ems* overlap to some extent. The *Tc-ems* and *Tc-otd* stripes show some small overlap throughout development (Figs. 4A–D).

In summary, the *Tribolium* orthologs are expressed in blastoderm stages in single stripes in the head anlagen but in contrast to *Drosophila* only *Tc-otd1* transcripts are detected in early blastoderm stages in a dynamic pattern indicative for early regionalization. *Tc-ems* and *Tc-btd* initiate later in already distinct stripes that do not cover more than one segment primordium. Hence, their expression patterns are not in line with a role in early regionalization events.

Divergent aspects of Tc-otd1 function correlate with changes of expression pattern

To identify the function of these genes in head development, we knocked down their transcripts using both parental (pRNAi) and embryonic RNAi (eRNAi). To verify that *Tc-otd2* does not contribute to head patterning, we knocked down its transcript via RNAi. Almost all embryos hatched and all showed the wild-type bristle pattern. The lack of an RNAi phenotype is not surprising regarding its late onset and restricted pattern of expression (Li et al., 1996). Therefore, we have excluded Tc-otd2 from further analysis.

The *Tc-otd1* phenotype has been described to range from a gap like deletion of ocular and antennal segments up to the deletion of the entire head (Schröder, 2003). We find this phenotypic range (Figs. 5A–F) but also detect stronger phenotypes in pRNAi experiments (Figs. 5G, H) confirmed by eRNAi performed 2–3 h after egg laying. Strong phenotypes lack not only the head, but also parts or the entire thorax (Figs. 5G, H). In about 20% of the RNAi treated embryos, we find cuticles with severe disturbances where only few thoracic and abdominal segments are left or even non-segmented sack-like cuticles with some residual bristles (not shown). A large portion of the RNAi phenotypes (63%) display posterior segmentation defects. The dorsal

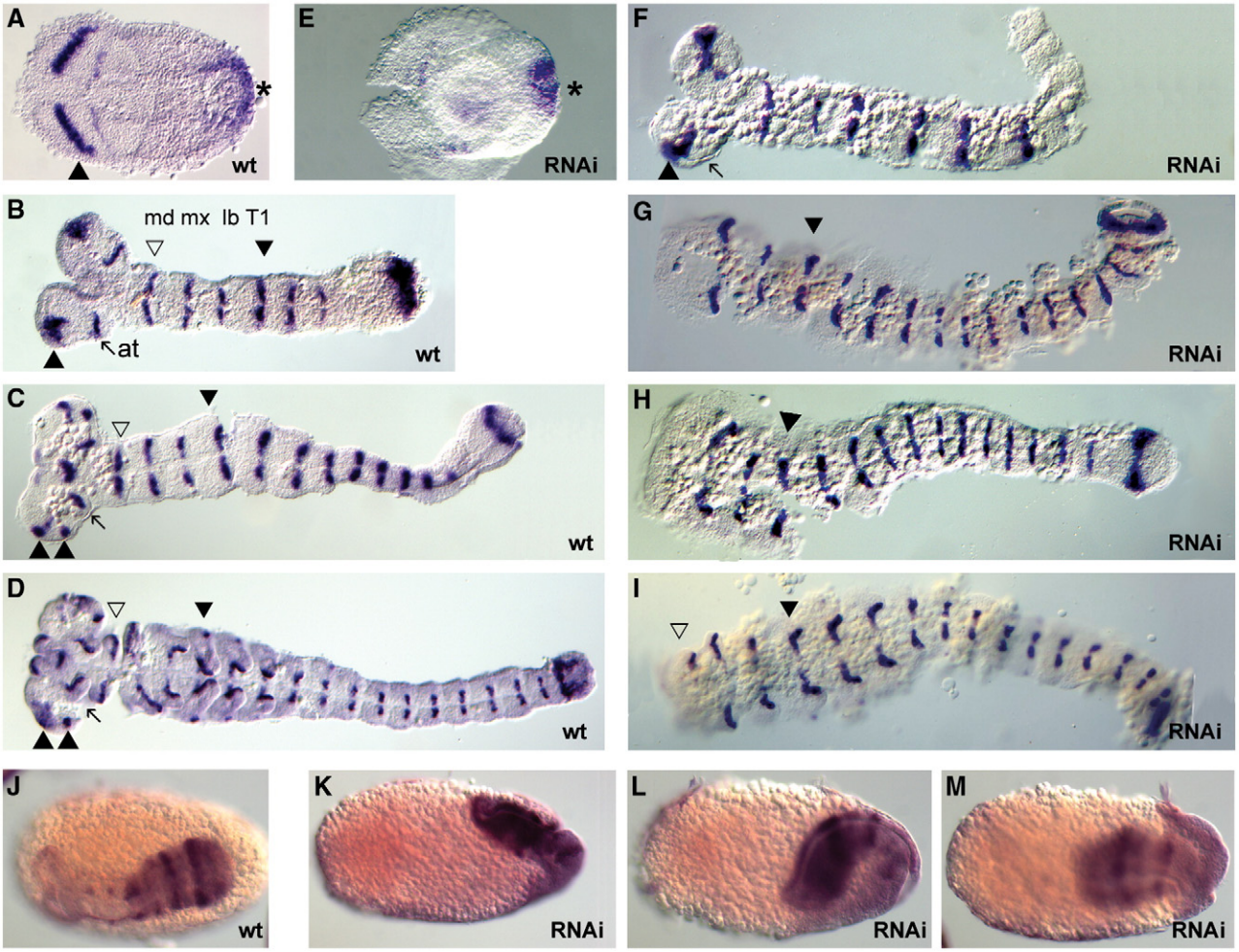

Fig. 7. *Tc-wg* in *Tc-otd1* RNAi knockdown embryos reflect the cuticular defects. All embryos are shown with anterior to the left. The mandibular segment is marked with an open arrowhead, the first thoracic segment with a black arrowhead. The ocular *Tc-wg* domain is marked with a black arrowhead while the antenna is indicated with an arrow. (A–D) Wild-type embryos at different stages of elongation. (E) Young germ band showing intact growth zone expression (star) but absent ocular *Tc-wg* domain. The head tissue appears to be less stable and deranged. (F) Weak phenotype that retains the ocular (arrowhead) but has lost the antennal *Tc-wg* domain (arrow). (G–I) Embryos in the extended germ band stage displaying different grades of anterior deletions of head and gnathal segments. (J–M) *Tc-hairy* staining in wild-type (J) and RNAi embryos (K–M) in approximately similar stages. The loss of head tissue leads to a drastic immersion of the growth zone into the yolk leading to a situation where the head remnants are at the posterior of the blastoderm while the posterior growth zone is directed toward anterior.

cuticle of thorax and anterior abdomen appear to be especially sensitive to *Tc-otd1* knockdown. In some cases, also more posterior segments of the abdomen are affected or deleted (not shown). In *Tribolium*, some strong segmentation or dorso-ventral phenotypes do not secrete cuticle. In order to assess in how far we miss potentially strong phenotypes in our cuticle analyses, we counted the portion of cuticles and empty egg shells after pRNAi. We find that both in wild-type and RNAi egg collections 30–38% of the eggs do not develop a cuticle which is a slightly elevated number. As we focus on head development in this work, we have not followed these defects further.

To map the head defects, we analyzed 24 cuticles that showed remnants of the head. Most of them (46%) lack all head structures but maxilla and labium (Fig. 5C). The maxilla appears more stable than the labium as it is often the only head segment present (29.1% versus 8.3%). Our data do not reveal if this is due to more stable patterning of the maxillary segment or to homeotic transformations. In 16.6%, the entire gnathum (mandible, maxilla and labium) is present while the antenna is missing (Fig. 5B). The non-segmental labrum remains present in 21% of the cuticles (Fig. 5A) either with our without antenna. To track down the weakest effects of *Tc-otd1* RNAi, we scrutinized 28 cuticles that displayed all head structures (labrum and all head appendages) for the bristle pattern described above (Table S1 and Figs. 5I, J). We find that the most sensitive region is marked by posterior and ventral vt, dorsal and posterior gt, the bell row bristle and the posterior and median maxilla escort bristles. Even in weak phenotypes, the eyes are usually absent. In stronger phenotypes, the defects extend toward anterior until they lead to loss of antenna, labrum and mandibles (see above). This type of deletion then usually includes loss of all vertex and gena bristles and the bell row. Interestingly, the weaker *Tc-otd1* RNAi phenotypes are similar to the *Drosophila* mutants where ocular and antennal structures are affected.

We wondered whether the maternal expression that is found in *Tribolium* but not *Drosophila* might be responsible for the strong phenotypes. To knock down the late Tc-Otd1 function without affecting the early maternal contribution, we injected embryos at different time points after egg laying and determined the portion of missing labrum, head and thoracic appendages in cuticle preparations. Indeed, the early injections (1–4 h after egg laying) lead to deletions of anterior segments up to the third thoracic segment while injections after 5 h elicit the weaker *Drosophila* like deletions (Fig. 6A). We were not able to stain injected blastoderm stages to determine the exact time point of transcript degradation upon RNAi and we do not know the dynamics of Tc-Otd1 protein turnover. Assuming a lag of 1–2 h, we estimate the more restricted requirement for *Tc-otd1* to start at 5–6 h. This analysis suggests that the *Tc-otd1* expression after retraction from both poles (Fig. 6B, 6–7 h) correlates with the more restricted RNAi phenotype (Fig. 6A, 5–6 h). From that time on, *Tc-otd1* expression is similar to *Drosophila* in location and extent and also the deletion domain of mutants/RNAi phenotypes covers approximately the same region (see Fig. 10).

To get more insight into the embryonic origin of the phenotype, we performed *Tc-wg* stainings in knockdown embryos. Essentially, the results reflect the defects seen in cuticles. In young embryos undergoing involution, the growth zone appears to be correctly specified and the posterior *Tc-wg* stripe is always present (black star in Figs. 7A, E). However, the anterior tissue is not properly formed and the ocular *Tc-wg* stripe is often missing (Fig. 7E). The loss of the entire head is reflected in deletions of the respective *Tc-wg* stripes in fully extended germ bands (Figs. 7G–I). We also find correlates for weaker deletions

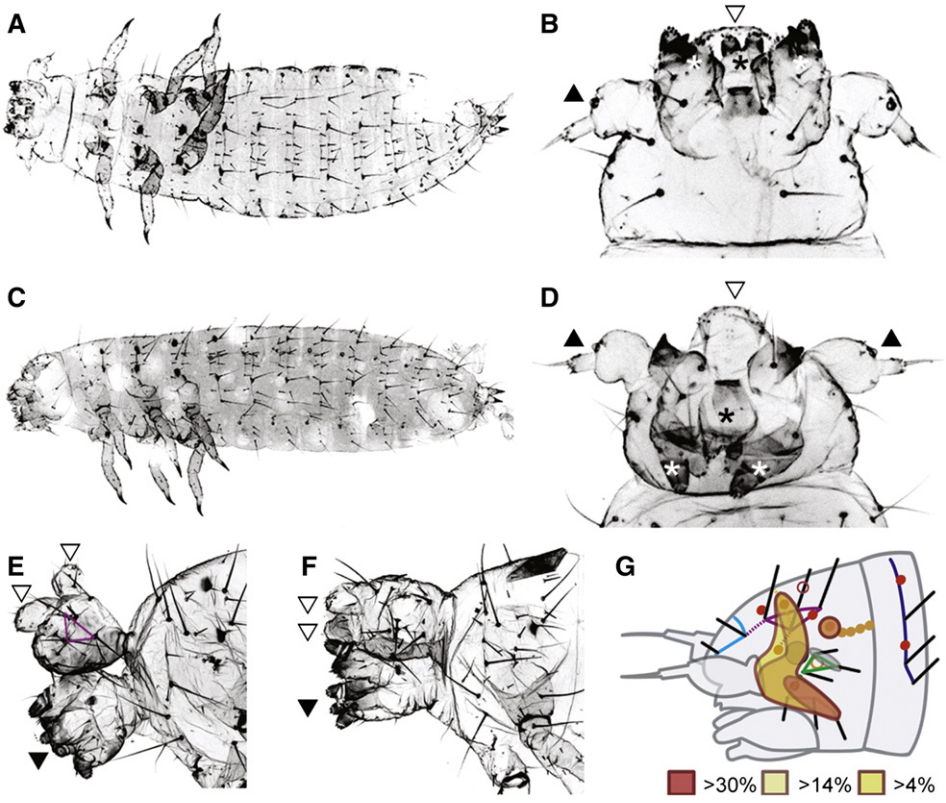

Fig. 8. *Tc-ems* RNAi leads to defects in the antennal segment. Knock down of *Tc-ems* function leads to mild phenotypes where the antennae are twisted toward posterior and are not properly formed at their basis. (A, C) Ventral and ventrolateral view of cuticles the head of which are shown in panels B and D. Labrum (open arrowhead), labium (black star) and maxillae (white stars) appear unaffected. (E, F) In rare cases, more tissue is deleted leading to embryos that have lost the antennae and remain with two separate lobes. A ventral lobe formed by the gnathal appendages (black arrowhead) and a dorsal lobe including dorsal cuticle and the labrum that is sometimes split (open arrowheads) and appears to contain the vertex triplet bristles (purple triangle). Because these defects occur rarely (3 cuticles of 23) and are not linked to the twisted antennae phenotype by intermediates, we assume that these defects are due to secondary morphogenesis defects. (G) The bristle pattern shows a deletion pattern partially overlapping with *Tc-otd1* (see Fig. 5). In addition, we find unusually frequent duplications of bristles in an additional more posterior region (>10% shaded in grey). Detailed results for the bristle analysis are found in Table S1.

as for instance the loss of the antennal stripe (Fig. 7F) or the antennal together with the mandibular stripes (not shown). Curiously, the deletion of anterior tissue apparently leads to an atypical twisting and immersion of the growth zone into the yolk (Fig. 7, compare K–M with J). In its extreme form, the twisting leads to an inversion of the embryonic axis relative to the egg axis (Fig. 7M). Curiously, a superficially similar rearrangement of the embryo within the egg is also observed in *Tc-zen1* RNAi (van der Zee et al., 2005). However, we do not see loss of serosa tissue in *Tc-otd1* RNAi (as judged by the wider spaced nuclei) but we observe rupturing of embryonic tissues anterior to the growth zone (not shown). An exact analysis of these early events is beyond the scope of this paper but apparently different kinds of changes in blastodermal tissue composition can lead to similar rearrangements of the growth zone within the egg.

Tc-ems function is restricted to the antennal segment

pRNAi and eRNAi knock down of *Tc-ems* leads to surprisingly mild cuticular phenotypes. The defect is marked by antennae with poorly formed basal segments that are posteriorly bent (Fig. 8) and the eyes are usually absent (not shown). The median and posterior bristles of the maxilla escort are most sensitive to *Tc-ems* RNAi. In stronger phenotypes, the defects extend dorsally including the eye, the ventral vt and median vertex bristle (Table S1 and Fig. 8F). In rare cases (14.3%), the antennae are lost together with a median portion of the head leading to a situation where a ventral lobe consisting of gnathal segments is separated from a dorsal lobe often consisting of a distally split labrum and other dorsal structures (Figs. 8E, F). The respective bristle pattern is strongly deranged and thus hard to be analyzed. The

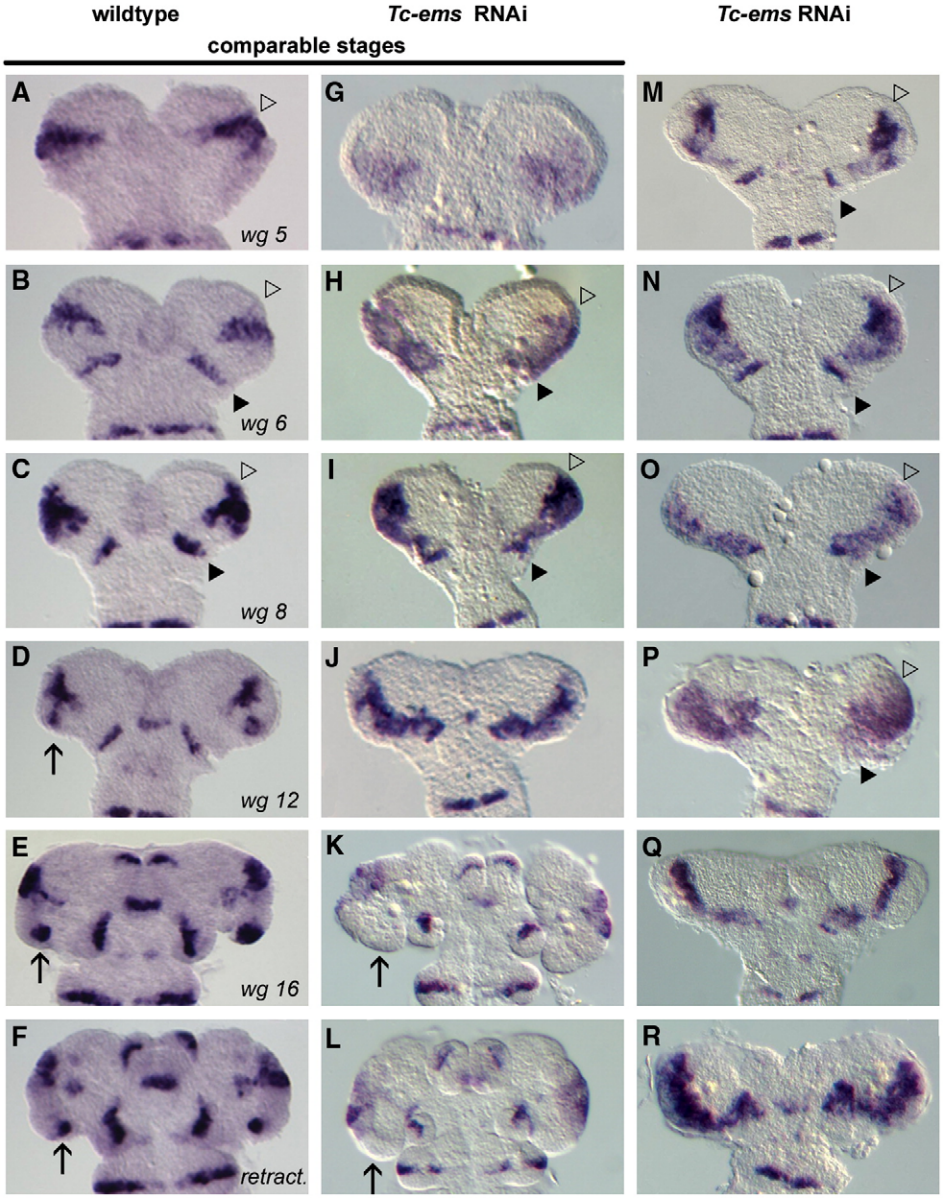

Fig. 9. *Tc-ems* determines the posterior border of the ocular *Tc-wg* domain. Heads of wild-type (A–F) and *Tc-ems* RNAi embryos (G–R). The age of embryos in panels G–L is comparable to the wild types panels A–F. The number of trunk *Tc-wg* stripes is given. Panels M–P are approximately the same stage but with different phenotypic strengths. The age compares to panels B and C. The age of the embryos in panels Q and R corresponds to panel D. The anterior border of the ocular *Tc-wg* domain is marked by an open arrowhead, the antennal stripe by a black arrowhead. (G–J) In early stages, the ocular *Tc-wg* domain is shifted posteriorly and is fused to the antennal *Tc-wg* domain. The posterior border of the antennal segment and the anterior border of the ocular segment appear to be unaffected (compare to panels A–D). (K, L) At later stages, the antennal stripe separates and becomes part of a shortened antenna. In wild type, the *Tc-wg* domain splits in two domains (E, F), the anterior of which is present in *Tc-ems* RNAi embryos but the posterior one is absent (compare arrows in panels E and F with panels K and L). (M, N) In weak phenotypes, the antennal *Tc-wg* stripe is unaffected and also the anterior border of the ocular *Tc-wg* domain appears normal (compare open arrowheads of panels B and C). (O–P) In stronger knockdowns, the ocular and antennal *Tc-wg* domain fuse and form one domain. (Q, R) This fused domain becomes a U shaped appearance in slightly older stages (comparable to panel D).

vertex setae, however, appear to be present (see purple triangle in Fig. 8E).

To get further insight into the embryonic development of the defect, we analyzed *Tc-wg* patterns in knockdown embryos (Fig. 9). We find that in weak phenotypes the posterior border of the ocular *Tc-wg* domain extends toward posterior while the antennal domain remains normal (Figs. 9I, M, N). In stronger phenotypes, these two domains fuse completely (Figs. 9H, O–R). Neither the anterior border of the ocular nor the posterior border of the antennal stripes appear to be affected although the highly dynamic ocular *Tc-wg* pattern makes it difficult to assess this unequivocally (compare Figs. 9A–F with G–L). Later, ocular and antennal domains appear to separate again (Figs. 9Q, R compare to D), giving rise to a distinct but shortened antennal *Tc-wg* stripe (Figs. 9K, L). Also the antenna itself is reduced and appears rounded rather then elongated (compare Figs. 9 K, L with E, F). In fully extended germ bands, the ocular *Tc-wg* domain splits into several domains, the posterior most of which probably marks the posterior boundary of the developing larval eye (Dong and Friedrich, 2005). This domain is clearly absent in *Tc-ems* RNAi embryos (black arrow in Figs. 9K, L and E, F) correlating with the absence of eyes in *Tc-ems* phenotypes. The anterior domain, in contrast, appears rather normal. Despite the segmentally iterated expression of *Tc-ems*, we do not find alterations of *Tc-wg* expression in the more posterior segments.

Tc-btd is not required for head development

Strikingly, we were not able to detect an RNAi phenotype for *Tc-btd*. To confirm this negative result, we stained knockdown embryos with a *Tc-btd* probe (including *Tc-caudal* as positive control in the same color reaction) and *Tc-wg*. Indeed, *Tc-btd* was knocked down below the limit of detection of whole mount *in situ* hybridization while both control staining and the *Tc-wg* pattern appeared normal (not shown). By counting laid eggs per female in comparison to a control (*Tc-ems* pRNAi), we confirmed that the injected beetles are not sterile and that a normal portion of embryos develops a cuticle (not shown). This suggests that the lack of cuticular phenotypes is not due to sterility of the injected animals or early embryonic death before the secretion of cuticle. In summary, we consider it likely that *Tc-btd* is not required for head development although we are aware of the inherent difficulty of proving the absence of gene function by RNAi (see materials and methods).

Discussion

Head development is not well understood in *Drosophila* because involution and derived morphology of the larval head have hampered the interpretation of mutant phenotypes. We have established the red flour beetle *Tribolium castaneum* as a model system for insect larval head development and asked how far the functions of the *Tribolium* orthologs of the known *Drosophila* head gap genes *otd*, *ems* and *btd* are conserved.

Tc-btd and Tc-ems are not head gap genes

Our most unexpected finding is that the *Tribolium* ortholog of *btd* is not required for head epidermis patterning. We did not see cuticular phenotypes although we were able to knock down *Tc-btd* below the detection limit of *in situ* hybridization. However, it is impossible to unequivocally confirm negative RNAi data as we cannot formally exclude that some Tc-Btd protein might have formed and been able to fully rescue a potential phenotype. Therefore, our interpretation remains arguable to some degree. However, the expression pattern supports our interpretation. In contrast to *Drosophila*, *Tc-btd* expression starts only at late blastoderm stages in a narrow stripe restricted to the future mandibular segment (Fig. 10). Only much later also antennal and intercalary stripes arise. This is not suggestive for an extensive early function in anterior head patterning. Interestingly, overexpression of *Dm-btd* in *Drosophila* does not lead to segmentation defects. This suggests that even in the fly the borders of *btd* expression are not instructive for metamerization (Wimmer et al., 1997). Our sequence analysis of the zinc finger and the buttonhead box has assigned clear orthology to Tc-SP8 and Tc-SP1234 and has revealed that the third factor (Tc-Btd) is the closest homolog of Dm-Btd in the *Tribolium* genome. However, these latter two genes do not cluster on one branch in the tree as would be expected of orthologs (Fig. S1D). The long branches of both genes indicate that they have undergone accelerated sequence evolution that has blurred their origin. Further support for our orthology statement comes from domain architecture, genomic location and late aspects of expression that are identical for both genes. Our notion that *btd* could be an *SP5* ortholog remains to be tested by the inclusion of *SP5*/*btd* orthologs from a broader arthropod sampling. But we note similarities of mouse *SP5* and *Tc-btd* in that both display dynamic embryonic expression patterns but lack overt phenotypes (Harrison et al., 2000; Treichel et al., 2001).

We find that *Tc-ems* is not a head gap gene because it is required only for the posterior portion of the ocular and the anterior portion of the antennal segment by positioning the posterior border of the ocular *Tc-wg* domain. Of the antenna itself, only the anterior most tissue is affected. This is likely to result in a one-sided disturbance of the outgrowth which in turn might lead to the bent antenna phenotype that we observe. The genesis of the strong phenotype with the dorso-ventrally split head remains obscure: we assume, however, that this is a secondary effect of loss of ocular and antennal tissue: The antennal tissue is located at the anterior end of the head that connects the upper head capsule with the gnathum below. Missing tissue there could lead to a loss of contact of the upper and lower part while both tissues continue development. Finally, both parts might separately close the “holes” that arise by the separation by formation of ectopic cuticle. Unfortunately, the morphogenetic movements that transform the head anlagen of the germ band to the final head are complicated and completely unknown so far—a closer description of these

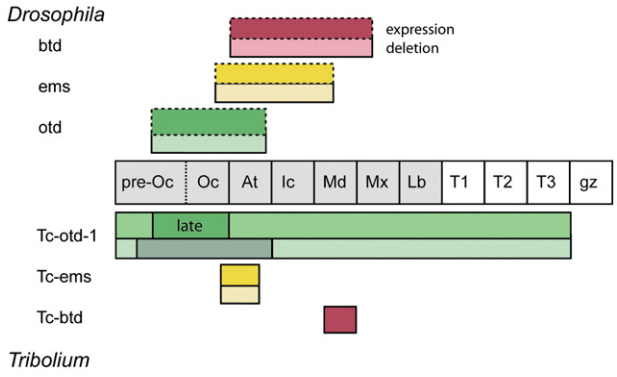

Fig. 10. Expression and deletion domains of *Drosophila* and *Tribolium otd*, *ems* and *btd* at early embryonic stages. Expression and deletion domains are aligned relative to the fate map of the embryo shown in the middle (pre-Oc: preocular region including labrum, segments: Oc: ocular, At: antennal, Ic: intercalary, Md: mandibular, mx: maxillary, Lb: labial, T: thoracic, gz: growth zone). The upper bars with more intense color indicate expression, the lighter bar below indicates the deletion domain in mutants or RNAi knockdowns. Expression of the *Drosophila* head gap genes is depicted as described for stage 5 embryos. They display overlapping expression and deletion patterns. However, the alignment of the expression domains to segment primordia has not been exactly determined and is based on the assumption that expression and deletion domains coincide (Wimmer et al., 1997) (hatched outline). The *Tribolium* orthologs are expressed in the same anterior posterior order but without significant overlap. The deletion domain of *Tc-ems* is restricted to the posterior part of the ocular and the anterior portion of the antennal segment while *Tc-btd* is not required for head development. *Tc-otd1* has an early broad expression domain that retracts to tissues including, and anterior to, the ocular segment (dark green). We find an early regionalization function that affects the entire blastoderm fate map and a later and more restricted head patterning function. The transition is apparently gradual. See text for more details.

processes is badly needed. In any case, the later and more restricted expression in the *Tribolium* blastoderm correlates with a narrower deletion domain when compared to *Drosophila* where *ems* is required for patterning the antennal, intercalary and parts of the ocular segment (Fig. 10).

Two phase function of Tc-otd1

Tc-otd1 is required for patterning large parts of the anterior embryo (Schröder, 2003). We find phenotypes that exceed the effects described before. They lack the entire head and thorax, show additional abdominal segmentation defects or in rare cases do not form segments at all. Interestingly, the severe deletions in embryonic injections only occur, when *Tc-otd1* dsRNA is injected within the first 4 h of development (at 32 °C) which correlates with the phase of broad and dynamic blastodermal expression of *Tc-otd1* (Fig. 6). We therefore suggest a two phase function of *Tc-otd1*: an "early regionalization function" and a later and more restricted "head patterning function". In the first phase, *Tc-otd1* could be required for partitioning the germ rudiment into non-growth zone versus growth zone tissue. In line with this model, we find phenotypes that lack all segments specified in the blastoderm (head and thorax) while the growth zone dependent abdominal segments are formed. The additional posterior patterning defects are unlikely to be direct effects, because *Tc-otd1* is not active at the posterior pole or in abdominal tissues at any time (apart from the midline expression). However, a massive loss of tissue in the anterior germ rudiment could affect growth zone integrity which secondarily could lead to posterior segmentation defects. Indeed we find that the growth zone is unnaturally turned and immersed into the yolk mass in knockdown embryos (Figs. 7K, L). A study of cell behavior and marker genes for the growth zone in knockdown embryos is required to test this hypothesis. The head patterning function of *Tc-otd1* is restricted to the ocular and antennal regions and hence is similar to the *Drosophila* function. One clear difference to *Drosophila* is the role of *Tc-otd1* for labrum development (Cohen and Jürgens, 1990; Finkelstein et al., 1990; Schröder, 2003). As the labrum anlagen are located in a tissue adjacent to *Tc-otd1* expression, the requirement could be indirect.

What is the ancestral state of *orthodenticle* expression? The late *otd* expression appears to be widely conserved among arthropods as for instance in *Parhyale hawaiiensis* (Crustacea) (Browne et al., 2006) and several chelicerates: the oribatid mite *Archegozetes longisetosus* (Telford and Thomas, 1998), the spider *Tegenaria saeva* and the scorpion *Euscorpius flavicaudis* (Simonnet et al., 2006). Also contribution to early regionalization is found throughout arthropods: Like in *Tribolium*, the hymenopteran *Nasonia vitripennis* ortholog is maternally expressed but in contrast to the beetle, its mRNA is localized to the anterior and also the posterior pole and plays important morphogenetic functions at both (Lynch et al., 2006). In the spider *Achaearanea tepidariorum*, *otd* is among the first genes to respond to the initial symmetry breaking event (Akiyama-Oda and Oda, 2003). Obviously, *otd* orthologs play a crucial role in early anterior patterning events in several arthropods but they perform in quite different ways. In contrast, *otd* expression in the scorpion *E. flavicaudis* starts at the 6 segment germ band stage in an already distinct anterior stripe arguing against a role at an earlier stage (Simonnet et al., 2006). Therefore, a requirement of *otd* for early anterior patterning is likely to be ancestral at least for holometabolous insects.

Different expression patterns correlate with different functions

In all three cases, we see a correlation of change in function with different expression patterns: The earlier and broader expression domains of *ems* and *btd* in *Drosophila* and *Tc-otd1* in *Tribolium* correlate with their requirement for patterning broader regions. This adds to the current view that *cis*-regulatory changes are crucial for the evolution of gene function (McGregor et al., 2007; Wray, 2007) and makes these genes interesting models to pin down the respective regulatory changes. Interestingly, it is the early aspect of expression that differs most between *Tribolium* and *Drosophila* while the late patterns appear conserved (Dalton et al., 1989; Finkelstein et al., 1990; Wimmer et al., 1993, 1996). There appears to be less constraint for evolutionary change of early patterning in insects. *orthodenticle* orthologs for instance have been found to be expressed zygotically (Cohen and Jürgens, 1990) or maternally, with either a ubiquitous (Li et al., 1996) or localized mRNA distribution (M.-F. Schetelig, B.G.M. Schmid, E.A.Wimmer, unpublished). Even mRNA localization to both poles has been found (Lynch et al., 2006) and a similar variety is seen for *giant* orthologs (Brent et al., 2007; Bucher and Klingler, 2004; Mohler et al., 1989). The lack of *bicoid* in most insects further supports the notion of highly evolvable early patterning in insects (Brown et al., 2001; Schröder, 2003).

Comparing gene function across bilaterian animals

The identification of genes that are involved in both vertebrate, annelid and insect anterior patterning has led to the suggestion of highly conserved mechanisms (Acampora et al., 1998; Denes et al., 2007; Reichert and Simeone, 1999; Simeone et al., 1992; Treichel et al., 2003; Wimmer et al., 1993). At first glance, the significance of such cross phylum comparisons is questioned by the variability that we detect even within holometabolous insects. However, we also show that this variability is mainly restricted to early patterning events while the late aspects of for instance *orthodenticle* expression are conserved between *Tribolium*, *Drosophila*, *P. hawaiiensis* (Crustacea) (Browne et al., 2006) and several chelicerates: the oribatid mite *A. longisetosus* (Telford and Thomas, 1998), the spider *T. saeva* and the scorpion *E. flavicaudis* (Simonnet et al., 2006). In the case of *ems*, the late expression pattern of insects is similar to the one in the spider *T. saeva* and the scorpion *E. flavicaudis* (Simonnet et al., 2006) and can therefore be regarded as the ancestral state. The comparisons between brain phenotypes in vertebrates and insects upon *ems* depletion, however, are currently based on *Drosophila* mutants that interfere with both early head gap gene patterning and later function in the brain (Hirth et al., 1995). Hence, these authors have potentially observed a composite phenotype where the early and broad requirement of *ems* in the ectoderm (that still comprises the neuroectoderm at that stage) may have produced a larger deletion of the brain than the later more restricted activity in the brain may do on its own. More specifically, the *Tribolium* phenotype that is restricted to the posterior portion of the ocular and anterior portion of the antennal segments indicates that only part of the deutocerebrum may actually depend on *ems* function rather than the entire deuto- and tritocerebrum (Hirth et al., 1995; Reichert and Simeone, 1999). We therefore argue that cross phylum comparisons should not be based on the extremely variable early blastodermal function and expression. In contrast, later aspects may tend to be more conserved because patterning then converges on the specification of organ primordia or even specific cell types with respective molecular fingerprints (Arendt, 2005; Tessmar-Raible et al., 2007) rather than the broad subdivision of embryonic fields.

This work shows that the head gap gene paradigm of *Drosophila* is not valid for other arthropods. Because of its insect typical mode of embryonic head development, *T. castaneum* is becoming the primary model system for head development. Its amenability to reverse genetics allows us to identify the crucial genes from an extensive candidate gene list (e.g. comprising genes involved in vertebrate neural plate patterning and/or genes expressed in relevant stages and tissues in *Drosophila*). In addition, forward genetics by the ongoing *GEKU* insertional mutagenesis screen (Göttingen, Erlangen, Kansas State University, USDA) will reveal novel players by a hypothesis independent approach. Finally, a detailed understanding of *Tc-ems*

and *Tc-otd1* function and their potential interaction with the dorso-ventral patterning system with its known effects on head development (van der Zee et al., 2006) is needed.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.03.005.

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